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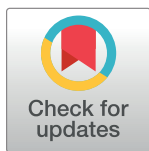
# Small RNAs and extracellular vesicles: New mechanisms of cross-species communication and innovative tools for disease control

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## Overview

Small RNA (sRNA)-mediated RNA interference (RNAi) is a conserved regulatory mechanism for gene expression throughout the domain Eukarya. Recent studies have shown that sRNAs can move between a host and an interacting organism to induce gene silencing *in trans*, a mechanism termed “Cross-Species RNAi” or, in many cases, “Cross-Kingdom RNAi.” Pathogens and parasites transport sRNAs into host cells during infection and silence host defense genes to suppress immunity, whereas hosts can also deliver their sRNAs into interacting microbes or parasites to suppress infection. Recent studies of different plant and animal hosts and their interacting organisms have unveiled extracellular vesicles (EVs) as vehicles of sRNA exchange in cross-species and cross-kingdom RNAi. The discovery of the pivotal role of sRNAs and EVs in cross-species and cross-kingdom communication offers innovative tools for pathogen and pest control in agriculture and biomedicine.

## Cross-kingdom RNAi

sRNAs—including microRNAs (miRNAs) that are processed by Dicer-like (DCL) proteins from single-stranded stem-loop-forming RNA precursors and small interfering RNAs (siRNAs) that are processed by DCL proteins from double-stranded RNA (dsRNA) precursors—are loaded into Argonaute (AGO) proteins to induce silencing of genes with complementary sequences [1]. Some sRNAs from diverse classes of pathogens and parasites are transported into host cells and induce cross-kingdom or cross-species RNA silencing to facilitate infection (Fig 1). Fungal pathogens, including ascomycete and basidiomycete species, can deliver sRNAs into their respective hosts [2–6]. In detail, *Botrytis cinerea*, the grey mold fungal pathogen that infects over 1,000 plant species, delivers sRNAs into plant cells and hijacks host RNAi machinery by loading its sRNAs into the *Arabidopsis* AGO1 protein to trigger silencing of host immunity genes, including mitogen-activated protein kinases (MAPKs), cell-wall-associated kinases, and other defense and signaling proteins [2]. A panel of sRNAs from *Verticillium dahliae*, which causes *Verticillium* wilt in many plant hosts, also move into plant cells and associate with the host AGO1 protein to silence host genes involved in plant defense [4]. A genome-wide association study shows that the white mold fungal pathogen *Sclerotinia sclerotiorum*

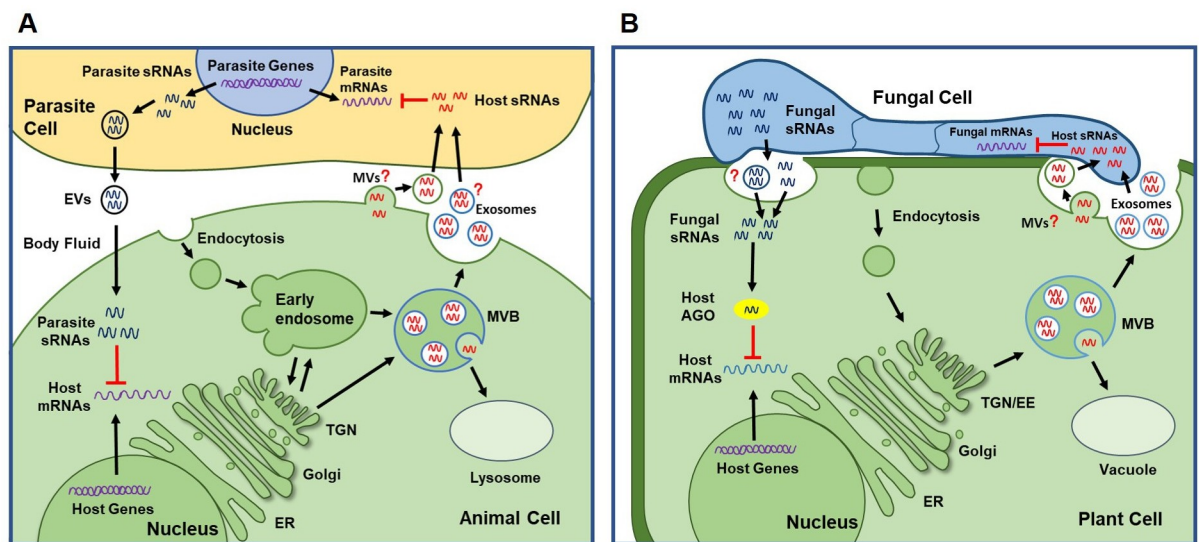
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produces sRNAs that, to facilitate infection, can target plant genes associated with quantitative disease resistance [5]. A miRNA-like sRNA from *Puccinia striiformis*, the causal agent of the destructive wheat stripe rust, targets wheat pathogenesis-related genes and suppresses host immunity to achieve successful infection [3]. Likewise, the parasitic plant *Cuscuta campestris* (dodder) transports several miRNAs into *A. thaliana* and *Nicotiana benthamiana* to promote invasion [7].

Cross-kingdom sRNA trafficking from a fungal pathogen to an animal host was also observed recently. *Beauveria bassiana*, an insect fungal pathogen, exports a miRNA-like RNA (bba-milR1) to the host mosquito, which induces cross-kingdom RNAi to suppress host immunity [6]. Strikingly, this insect fungal pathogen-derived bba-milR1 also binds to host AGO1 and silences mosquito target gene Toll receptor ligand Spätzle 4 [6], which is consistent with the mechanism used by transported sRNAs from plant fungal pathogens [2, 4].

In addition to eukaryotic pathogens, prokaryotic microbes can also use cross-kingdom RNA trafficking to manipulate gene expression in the hosts. Specifically, the root-nodule bacterium *Rhizobium* delivers tRNA-derived sRNA fragments (tRFs) into soybeans to suppress host genes involved in nodule formation and root development, which enhances nodulation efficiency [8]. Surprisingly, these *Rhizobium* tRFs also function through host AGO1 [8], just like fungal pathogen-derived sRNAs that are bound with host AGO1 to silence host target genes [2, 4, 6]. Furthermore, it has long been known that virus- or viroid-derived sRNAs can target various host protein-coding genes to facilitate infection in both plant and animal hosts [9–14]. A recent study revealed that the targeting of a long noncoding RNA in tomato by tomato yellow leaf curl virus-derived sRNAs contributes to disease symptoms [15].

Cross-kingdom RNAi is bidirectional. Plant hosts also transport sRNAs into fungal pathogens to suppress the expression of virulence-related genes, which contributes to plant defense



**Fig 1. Cross-species and cross-kingdom RNAi between host and coinhabitants.** (A) Cross-species RNAi between mammals and parasites. Parasites produce EVs containing parasitic sRNAs, which are internalized by mammalian cells to silence host genes involved in inflammation and innate immunity. Animal cells can deliver sRNAs into interacting organisms. They also secrete EVs (e.g., exosomes or MVs) containing host sRNAs. It is likely that animal hosts may also transport sRNAs using EVs into parasites to suppress parasitic genes. (B) Cross-kingdom RNAi between plants and fungal pathogens. Fungal sRNAs translocate into plant cells and hijack host AGO protein of the RNAi machinery to suppress plant immune responses. It is still unclear how pathogens transport sRNAs. Conversely, plants secrete EVs to transport host sRNAs into pathogens to silence fungal genes involved in virulence. The “?” indicates a prediction that has not been validated experimentally. AGO, Argonaute; EE, early endosome; ER, endoplasmic reticulum; EV, extracellular vesicle; MV, microvesicle; MVB, multivesicular body; RNAi, RNA interference; sRNA, small RNA; TGN, trans-Golgi network.

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responses. Translocation of plant endogenous sRNAs into fungi was clearly demonstrated by sRNA profiling of fungal cells purified from infected plant tissue [16]. Cai and colleagues developed an innovative sequential protoplastation method, which allowed for the removal of all plant cells and the purification of *B. cinerea* protoplasts/cells from infected *Arabidopsis* tissue [17]. These purified fungal cells contain host miRNAs and siRNAs, including *Trans*-acting siRNAs, also called secondary phasing siRNAs (phasiRNAs) [16]. These *Arabidopsis* sRNAs are delivered into interacting *B. cinerea* cells to induce silencing of fungal genes that are involved in pathogenicity, many of which are related to vesicle trafficking [16]. Mutated *B. cinerea* strains with a deletion in these target genes displayed reduced pathogenicity on plant hosts [16]. Another study found that cotton miRNA166 and miRNA159 accumulated in the mycelium of *V. dahliae* grown on artificial agar medium 30 days post re-isolation from infected tissue, which suggests that cotton miRNAs can translocate into *V. dahliae* [18]. Both cotton miRNAs trigger silencing of *V. dahliae* genes involved in virulence, *Ca*<sup>2+</sup>-dependent cysteine protease (*Clp-1*), and *isotrichodermin C-15 hydroxylase (HiC-15)*, which enhances disease resistance against this vascular pathogen [18]. Similarly, the wheat miRNA1023 suppresses an *alpha/beta hydrolase* gene in *Fusarium graminearum*, which is important for fungal infection [19]. Plant sRNA-induced silencing of pathogen genes is not restricted to fungi. A similar phenomenon was later observed in the interaction between plants and an oomycete pathogen, *Phytophthora capsici*. *Arabidopsis* may use secondary sRNAs to silence *Phytophthora* genes during infection [20].

Cross-species RNAi also exists in animal–parasite interactions. Some mammalian parasites use cross-species RNAi strategies to silence host genes and enable infection. For instance, the gastrointestinal nematode *Heligmosomoides polygyrus* (also known as *H. bakeri*) secretes sRNAs, including miRNAs, which suppress type II innate immune response in the murine host [21]. Conversely, some animal hosts also deliver sRNAs into parasites. Patients who suffer from sickle cell anemia show abnormal erythrocyte development but exhibit resistance to the malaria parasite *Plasmodium falciparum*. One of the reasons for malaria resistance is that these patients accumulate higher levels of a specific panel of miRNAs, which are transported into the parasite and suppress *P. falciparum* virulence [22]. Though *P. falciparum* lacks canonical RNAi components, such as DCLs and AGOs, the authors demonstrated that cross-kingdom RNA regulation occurs through impaired ribosomal loading by the fusion of host miRNAs with the parasite target mRNAs. This chimerization blocks target mRNA translation and causes an inhibition of parasite growth [22]. Anti-*Plasmodium* cross-kingdom RNA regulation was also reported based on the human miR-451/140 targeting the *P. falciparum* *antigen erythrocyte membrane protein-1 (PfEMP1)*. Human miR451 was found in the parasitic cell in complex with human AGO2, providing the first example of cross-kingdom delivery of an sRNA–AGO complex [23].

In the mammalian gut, miRNAs secreted by human and mouse intestinal epithelial cells were shown to influence gene expression even in gut bacteria that lack canonical RNAi machinery, suggesting a regulatory role of host miRNAs in gut microbiome homeostasis [24]. Furthermore, dietary plant miRNAs can also enter gut bacteria through plant-derived exosome-like nanoparticles, further shaping the gut microbial community [25]. RNAi does not exist in prokaryotes per se; however, bacteria have various ribonucleases, including type III ribonucleases [26], which may interact with the host or dietary miRNAs to interfere with bacterial mRNA expression. The increasing number of discovered cases of cross-species and cross-kingdom RNAi or RNA *Trans*-regulation across diverse host–microbe and host–parasite systems has made it clear that cross-species and cross-kingdom RNA communication is likely a ubiquitous mechanism in host–microbe and host–parasite interactions.

## EVs in animal–parasite interactions

In mammals, RNAs circulating through body fluids are often encapsulated in extracellular vesicles (EVs). EVs are membrane-surrounded vesicular compartments released by cells to the extracellular environment to transport proteins, RNAs, lipids, and other molecules to other cells or to interacting organisms [27]. EVs are categorized into multiple classes based on their biogenesis pathways and associated protein markers. In mammalian systems, multiple classes of EVs have been shown to carry sRNAs. In particular, exosomes, which are derived from multivesicular bodies (MVBs) and have tetraspanin proteins as one of the key protein markers [28], play an important role in sRNA trafficking [29]. Microvesicles, which bud from the plasma membrane, can also transport sRNAs into recipient cells [30]. Both types of EVs are involved in cell-to-cell communication in homeostasis, immune signaling, and neural networks [31, 32]. While exosomes and microvesicles are secreted during normal cellular processes, apoptotic bodies are formed during programmed cell death [33]. Functional molecules, including RNAs, can be detected in apoptotic bodies [34, 35]. Some reports have shown that apoptotic bodies can transport these functional molecules into recipient cells [35, 36], though whether they are also involved in cross-kingdom communication between parasites/microbes and animal hosts remains to be explored.

It is not surprising that pathogens and pests would evolve to exploit or target these natural cell-to-cell communication pathways. Diverse parasites have been shown to use EVs to deliver sRNAs to host cells and modulate host gene expression (Fig 1A) [37]. The miRNA-containing EVs that are released by the gastrointestinal nematode—or helminth—*H. polygyrus* are internalized by host mouse cells and suppress inflammation and innate immune responses during infection [21]. Many of the nematode miRNAs share common ancestry and identical seed sites with miRNAs of the mouse host, such that they would be expected to be able to tap into existing miRNA target networks in the mouse cell. However, the RNAi mechanisms used between these two animals are complex, as the nematode packages a nematode-specific AGO protein (extra cellular worm Argonautes [exWAGO]) into the EVs bound to siRNAs from rapidly evolving nongenic regions of the parasite genome [38]. Indeed, these studies suggest that different parasites and pathogens might have diverse tools for RNA-mediated suppression of host genes. The study of these pathogen RNA transmission mechanisms may guide new strategies for effective therapeutic delivery of RNAs (for example, delivering RNA–AGO complexes, rather than RNA alone) [39]. Since the EVs from helminths are immune suppressive, the EVs and their RNA cargoes also represent another potential therapy for treating colitis and allergies in humans [21, 40, 41].

In mammalian systems, EVs have been shown to transport sRNAs between cells within the organism; we speculate that EVs may also be used by the host cells to deliver sRNAs to its interacting organisms, such as parasites and pathogens.

## EVs in plant–microbe interactions

In 1967, plant EVs were initially observed in carrots by electron microscopy [42]. Forty years later, Regente and colleagues isolated plant EVs from extracellular wash fluids of imbibed sunflower seeds [43]. However, the origin of plant EVs still remained unknown. In mammals, exosomes are a class of EVs derived from MVBs. Mammalian tetraspanins cluster of differentiation (CD)63, CD81, and CD9 are enriched in exosome membranes and are commonly used as biomarkers to isolate and phenotype exosomes [28]. *Arabidopsis* encodes 17 members of the *TETRASPANIN* (*TET*) family [44], and two *Arabidopsis* *TET*s (*TET8* and *TET9*) are induced upon infection by *B. cinerea*. Moreover, *TET8*-associated vesicles accumulated to a high level at the fungal infection sites [16]. *TET8* is colocalized with *Arabidopsis* MVB-marker Rab5-like



GTPase ARA6 inside the cell, and TET8-associated vesicles are secreted into the apoplast [16], suggesting that TET8-associated EVs are derived from MVBs and secreted into apoplastic space and can, therefore, be considered bona fide plant exosomes. These exosomes contain plant-endogenous sRNAs and are efficiently taken up by *B. cinerea* fungal cells. Plant exosomes deliver sRNAs into fungal pathogens to suppress fungal infection by inducing silencing of fungal virulence-related genes. Similarly, *Arabidopsis* also transports secondary phasiRNAs from PPR gene clusters into an oomycete pathogen, *P. capsici*, likely also by EVs, which silence target genes in the pathogen [20]. Thus, plants have adapted EV-mediated cross-kingdom RNAi for immune responses during the coevolutionary arms race with interacting pathogens (Fig 1B).

In addition to exosomes, PENETRATION (PEN)1-associated EVs, which contain several stress-response-related proteins, were identified in *Arabidopsis* [45]. The biogenesis pathway of PEN1-associated EVs remains unclear, although PEN1 was originally identified as a plasma-membrane-associated plant-specific syntaxin [46]. PEN1-associated EVs were purified from the apoplast wash fluid of *Arabidopsis* leaves using an ultracentrifugation speed (40,000g) [45, 47], which is slower than that used to isolate TET-associated exosomes (100,000g) [16]. Secretion of PEN1-associated EVs was increased during infection by a bacterial pathogen (*Pseudomonas syringae*) or following treatment with the phytohormone salicylic acid [45]. Baldrich and colleagues analyzed the sRNA population in these EVs isolated from uninfected *Arabidopsis* leaves and found that these EVs carry predominantly “tiny RNAs,” which are 10–17 nucleotides in length and derived mainly from the positive strand of mRNA transcripts [48]. It is not clear whether these tiny RNAs have any biological function. Since pathogen-infected samples were not included in this study, whether this class of EVs is also involved in plant and pathogen interactions and whether tiny RNAs are delivered into pathogen cells via these EVs remain unclear. PEN1 and the ATP-binding cassette (ABC)-transporter PEN3 are incorporated into extracellular encasements surrounding the haustoria of the powdery mildew fungus, *Golovino-mycetes orontii*, suggesting that PEN1-associated EVs contribute to defense responses against powdery mildew [45, 49, 50]. A third type of plant EV, which is derived from a novel double-membrane-bound exocyst-positive organelle (EXPO) [51], has been reported in plants. These EXPO-derived EVs were discovered through transient expression of exocyst subunit exo70 family protein E2 (Exo70E2), a component of exocyst complex, in protoplasts from *Arabidopsis* suspension-cultured cells. Whether EXPO-derived EVs contain RNAs and are involved in cross-kingdom communication remains to be discovered.

Similar to animal EVs, which comprise diverse, heterogeneous, and cell-type-specific populations with a wide range of biological functions in cell-to-cell communication [52], the previously cited studies suggest that plant cells also secrete different classes of EVs that may contain specific cargoes. Establishing plant EV biomarkers (such as TET8, PEN1, and Exo70E2) will enable immuno-based analysis of EVs to further understand the biological functions of EVs in complex biological systems such as plant-microbe interactions.

Though EV-mediated transport is a key mechanism for RNA secretion and delivery between hosts and microbes/pests, nonvesicular extracellular RNAs have also been discovered. Specifically, in human plasma, extracellular RNAs were found within RNA-protein complexes, including AGO proteins and high-density lipoprotein complexes [53–56]. Additionally, exomeres, extracellular nonmembranous nanoparticles, have recently been discovered in mammalian systems containing AGO1, AGO2, and AGO3 proteins; amyloid precursor proteins; RNAs; and DNAs. Notably, these exomeres contained a profile of macromolecules distinct from exosomes [57, 58]. In a plant system, Baldrich and colleagues found that sRNAs were still present in apoplastic wash fluid, which they believe was depleted of EVs by centrifugation at 40,000g [48]. However, small, RNA-containing EVs, such as exosomes, are mostly

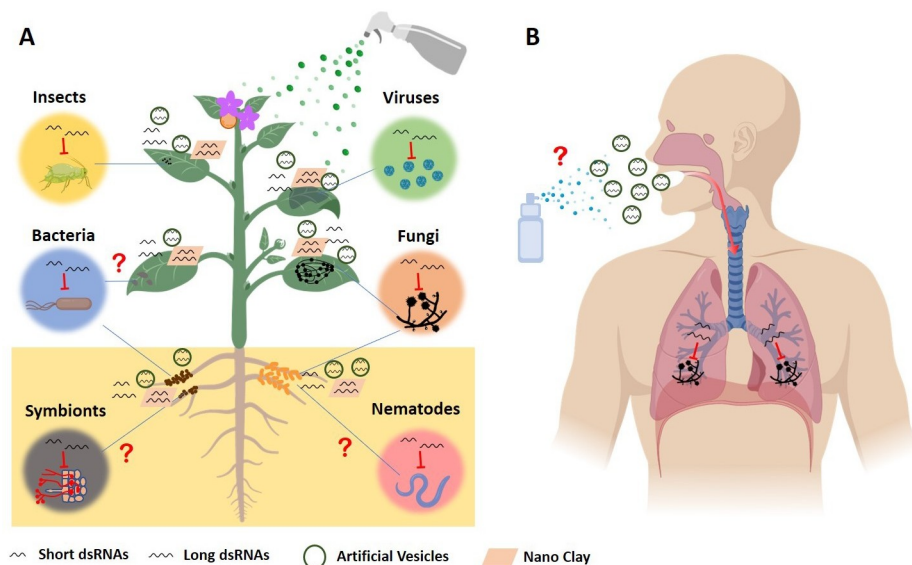
collected at higher speeds (between 100,000g and 120,000g) from various plant and mammalian systems [52, 59–61], as well as from fungi [62, 63]. Furthermore, plant tetraspanin-labeled exosomes, which transport sRNAs from the hosts to fungal cells, were much more enriched after centrifugation at a speed of 100,000g than at 40,000g [16]. Therefore, it is unlikely that plant EVs can be depleted at 40,000g, and, consequently, whether nonvesicular RNAs are secreted by plants requires further investigation. Furthermore, the origins of nonvesicular RNAs and their potential role in cross-kingdom RNAi remain to be explored.

## RNA and EV-based innovative tools for disease control

Global disease control mainly relies on chemical protection measures using fungicides, pesticides, and antibiotics, which not only threatens the health of humans and ecosystems but also generates novel uncontrollable drug-resistant pathogenic strains [64]. We are in urgent need of innovative, durable, and eco-friendly fungicides and antimicrobial drugs to avoid a global collapse in our ability to control pathogen/parasite infections in both plants and animals, including humans.

One direct application of cross-kingdom RNAi is host-induced gene silencing (HIGS), a promising technology in which transgenic plants express dsRNAs or sRNAs that target pathogen or insect virulence-related genes to combat plant diseases [65, 66]. This approach has also made it possible to control multiple pathogens spontaneously by designing dsRNA and sRNA constructs that target multiple genes from different pathogens [4]. Although HIGS is effective, it involves the generation of genetically modified organisms (GMOs), which is not only technically challenging in many crop species but unfortunately still a concern for many consumers. Furthermore, GMOs are banned in European agricultural productions, rendering HIGS not practically usable, at least in the near future.

Environmental RNAi, initially discovered in the nematode *Caenorhabditis elegans* [67], is the cellular uptake of RNAs from the environment and the induction and spreading of systemic gene silencing. Forward genetics screening in *C. elegans* revealed that *Systemic RNA interference deficient (SID)-1* and *SID-2* encode for two dsRNA transmembrane channel proteins, which are required for dsRNA uptake and systemic gene silencing [68, 69]. In this invertebrate system, there is higher uptake and silencing efficiency for long dsRNA (>60 bp) than short (<25 bp) or single-stranded RNA [70, 71]. Inspired by environmental RNAi of *C. elegans*, Wang and colleagues tested whether fungal cells can also take up RNAs from the environment and observed rapid RNA uptake by *B. cinerea* cells [4]. These RNAs induce silencing of fungal genes in a sequence-specific manner. Unlike *C. elegans*, which primarily takes up long dsRNAs, fungal uptake of environmental RNAs seems less dependent on RNA size, because both short sRNA duplexes and long dsRNAs are taken up by fungi and induce robust gene silencing in the fungal cells [4]. Fungal environmental RNAi allowed plant scientists to design spray-induced gene silencing (SIGS) to control fungal and potentially other pathogens through spray application of pathogen gene-targeting dsRNAs and sRNAs (Fig 2A) [4, 72, 73]. Wang and colleagues demonstrated that spray application of long dsRNAs or sRNA duplexes that target *B. cinerea* *DCL1* and *DCL2* genes can effectively suppress grey mold diseases on fruits, vegetables, and flowers [4]. Koch and colleagues have shown that SIGS can also effectively control a fungal disease in the monocot barley [73]. Spray application of a long dsRNA that targets fungal cytochrome P450 *lanosterol C-14 $\alpha$ -demethylase* genes on barley leaves can inhibit *F. graminearum* infection [73]. Similarly, application of exogenous dsRNAs helps protect *Brassica napus* from infection by *S. sclerotiorum* and *B. cinerea* [74]. These pathogen gene-targeting dsRNAs and sRNAs represent a novel class of eco-friendly fungicides, “RNA fungicides” (Fig 2A).



**Fig 2. SIGS is an efficient disease control strategy in plants and potentially in humans.** (A) Spray application of dsRNAs and sRNAs that target pathogen/pest genes can potentially control plant diseases. The SIGS-based protection can be prolonged by incorporating RNAs into artificial vesicles (black circle) or nanoparticles (pink rhombus) to protect RNAs from degradation or water rinsing. (B) Future RNA-based antifungal drugs have the potential to control human mycoses. Artificial vesicles/liposomes will likely facilitate the RNA delivery. Figures were created with BioRender. The “?” indicates a prediction that has not yet been validated experimentally. dsRNA, double-stranded RNA; SIGS, spray-induced gene silencing; sRNA, small RNA.

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Exogenous RNAs can either be directly internalized into fungal cells [4] or indirectly via passage through plant tissue before transport into interacting pathogen cells [75]. Furthermore, Koch and colleagues observed inhibition of *F. graminearum* growth in the distal nonsprayed barley leaf tissue [73], suggesting that sprayed dsRNAs taken up by plant cells moved through vasculature systemically. While the molecular mechanism of RNA uptake in *C. elegans* and some nematodes is based on SID proteins, which are not present in plants or fungi, the mechanisms for uptake of environmental RNAs into fungi and plants need further investigation.

Obviously, the effectiveness of SIGS relies on extracellular RNA stability and RNA uptake efficiency of pathogens. To technically improve RNA stability, Mitter and colleagues docked an antiviral dsRNA onto double hydroxide clay nanosheets, which increased the efficacy of plant antiviral protection [75]. In addition, the use of artificial vesicles or liposomes to protect RNAs could be an effective strategy to improve SIGS for plant protection and to develop potential antifungal drugs for therapy, as some fungi are capable of taking up EVs efficiently (Fig 2) [16, 76]. Since EV trafficking is also a natural RNA transport mechanism in mammals, it is exciting to consider the potential for extension of artificial vesicle-protected RNA-based antifungal strategies in humans (Fig 2B). Indeed, lipid-based nanoparticles have been used to stabilize therapeutic compounds, including sRNAs, in biomedical applications [77]. For example, liposomal amphotericin B, the world's leading antifungal drug, was based on liposomal formulation of amphotericin B to reduce toxicity [78]. Moreover, Walker and colleagues have observed that amphotericin B-containing liposomes remained intact during transit through the cell walls of phylogenetically distant fungal pathogens, *Candida albicans* and *Cryptococcus neoformans*, although liposomes (60–80 nm) are larger than the theoretical cell wall porosity



(approximately 5.8 nm)[79]. This work suggests that the fungal cell wall is deformable and viscoelastic to allow liposomes to pass, which makes it possible to efficiently deliver new generation of antifungal drugs, including RNA-based drugs, using liposomes/artificial vesicles [79]. In 2018, the Food and Drug Administration (FDA) approved the very first therapeutic siRNA drug, patisiran, to treat hereditary transthyretin-mediated amyloidosis, a rare, debilitating and often fatal genetic disease [80]. Patisiran uses a lipid nanoparticle delivery mechanism to transfer 21-bp siRNA duplex into cells in the liver [80]. Besides patisiran, there are at least 6 other RNAi therapeutics already in phase III clinical trials [80].

Although more than 300 human or animal pathogenic fungal species have been recorded and fungal infections display disproportional high mortality rates, mycoses are rather neglected in infection biology research [81]. Survival rates of patients suffering from respiratory and systemic fungal infections often caused by the opportunistic fungi *Candida* (candidiasis), *Aspergillus* (aspergillosis), or *Cryptococcus* (cryptococcosis) are low due to limited availability of antifungal drugs. Drug-resistant fungal strains have already emerged to all the commonly used antifungal drugs [64]. Therefore, innovative drugs to combat fungal infections are urgently needed, and based on the effects observed for antifungal SIGS approaches in plants, development of novel antifungal RNA therapeutics and artificial vesicle/liposome-mediated delivery methods may be effective in the fight against mycoses.

## Future perspectives

The field of cross-species and cross-kingdom communication via RNA is still in its infancy, yet an increasing number of studies across diverse systems demonstrate that mobile RNAs are key regulatory molecules that shape the interactions between hosts and interacting pathogens or organisms. Plants and animals deliver sRNAs into interacting (micro-)organisms to inhibit infection, and pathogens and parasites can, in turn, transport sRNAs into the host to suppress host immunity. Current studies show that EVs play an essential role in transporting sRNAs from the plant hosts to pathogens and from parasitic nematodes to mammalian hosts, and it is very likely that mammalian hosts could also utilize EVs to deliver sRNAs into their parasites and pathogens, though this is currently just speculation. Recent advances in methodology development for isolating different classes of EVs in mammalian systems provide excellent tools and guidelines to study RNA delivery in cross-species and cross-kingdom RNAi [82, 83]. Although there is diversity in the properties of EVs based on their cell and tissue origin (and purification techniques, which can also impact the exact profile of RNAs and proteins found in EVs), it is clear that small EVs, including exosomes, play an important role in delivering sRNAs [60, 84, 85].

The discovery of cross-species and cross-kingdom RNAi and fungal RNA uptake has inspired scientists to design novel disease control strategies against pathogens and pests in agriculture, such as HIGS and SIGS. Structural and mechanistic studies of EVs in sRNA trafficking allows for the development of innovative delivery methods of sRNAs using artificial vesicles, or nanoparticles, which may also be considered for therapeutic applications in mammalian systems. We speculate that future development and application of a new generation of RNA-based fungicides and antifungal drugs will be an important research direction to control diseases caused by eukaryotic pathogens and parasites.

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